SEQ ID NO: 63 is the amino acid sequence identified as "aab95283" in Figure 6A-6N.

SEQ ID NO: 64 is the amino acid sequence identified as "aab97723" in Figure 6A-6N.

SEQ ID NO: 65 is the amino acid sequence identified as "aac17079" in Figure 6A-6N.

SEQ ID NO: 66 is the amino acid sequence identified as "aac18062" in Figure 6A-6N.

SEQ ID NO: 67 is the amino acid sequence identified as "aac27152" in Figure 6A-6N.

C1

SEQ ID NO: 68 is the amino acid sequence identified as "aac99311" in Figure 6A-6N.

SEQ ID NO: 69 is the amino acid sequence identified as "aad12025" in Figure 6A-6N.

SEQ ID NO: 70 is the amino acid sequence identified as "caa20531" in Figure 6A-6N.

SEQ ID NO: 71 is the amino acid sequence identified as "caa64636" in Figure 6A-6N.

SEQ ID NO: 72 is the amino acid sequence identified as "caa94432" in Figure 6A-6N.

SEQ ID NO: 73 is the amino acid sequence identified as "cab06427" in Figure 6A-6N.

SEQ ID NO: 74 is the amino acid sequence identified as "cab10318" in Figure 6A-6N.

Please replace the paragraph at page1, lines 8-9 with the following:

C2

This application is a divisional of U.S. Application No. 09/457,046, filed December 7, 1999, now issued as U.S. Patent No. 6,287,835, herein incorporated by reference, which is a continuation in part of U.S. Application No. 09/411,145, filed September 30, 1999, now abandoned, herein incorporated by reference.

Please replace the paragraph at page 2, lines 23-28 with the following:

C3

The second specific step of Taxol™ biosynthesis is an oxygenation reaction catalyzed by taxadiene-5α-hydroxylase (**Figure 1**). The enzyme, characterized as a cytochrome P450, has been demonstrated in *Taxus* microsome preparations to catalyze the stereospecific hydroxylation of taxa-4(5),11(12)-diene, with double bond rearrangement, to taxa-4(20),11(12)-dien-5α-ol (Hefner et al., *Chem. Biol.* **3**:479-489, 1996).

Please replace the paragraph at page 2, line 29 through page 3, line 16 with the following:

C4

The third specific step of Taxol™ biosynthesis appears to be the acetylation of taxa-4(20),11(12)-dien-5α-ol to taxa-4(20),11(12)-dien-5α-yl acetate by an acetyl CoA-dependent transacetylase (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999), since the resulting acetate ester is then further efficiently oxygenated to a series of advanced polyhydroxylated

Taxol™ metabolites in microsomal preparations that have been optimized for cytochrome P450 reactions (**Figure 1**). The enzyme has been isolated from induced yew cell cultures (*Taxus canadensis* and *Taxus cuspidata*), and the operationally soluble enzyme was partially purified by a combination of anion exchange, hydrophobic interaction, and affinity chromatography on immobilized coenzyme A resin. This acetyl transacylase has a pI and pH optimum of 4.7 and 9.0, respectively, and a molecular weight of about 50,000 as determined by gel-permeation chromatography. The enzyme shows high selectivity and high affinity for both cosubstrates with K_m values of 4.2 μM and 5.5 μM for taxadienol and acetyl CoA, respectively. The enzyme does not acetylate the more advanced Taxol™ precursors, 10-deacetylbaccatin III or baccatin III. This acetyl transacylase is insensitive to monovalent and divalent metal ions, is only weakly inhibited by thiol-directed reagents and Co-enzyme A, and in general displays properties similar to those of other *O*-acetyl transacylases. This acetyl CoA:taxadien-5α-ol *O*-acetyl transacylase from *Taxus* (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999) appears to be substantially different in size, substrate selectivity, and kinetics from an acetyl CoA:10-hydroxytaxane *O*-acetyl transacylase recently isolated and described from *Taxus chinensis* (Menhard and Zenk, *Phytochemistry* **50**:763-774, 1999).

C4

Please replace the paragraph at page3, lines 17-20 with the following:

Acquisition of the gene encoding the acetyl CoA:taxa-4(20),11(12)-dien-5α-ol *O*-acetyl transacylase that catalyzes the first acylation step of Taxol™ biosynthesis and genes encoding other acyl transfer steps would represent an important advance in efforts to increase Taxol™ yields by genetic engineering and *in vitro* synthesis.

C5

Please replace the paragraph at page 6, lines 5-6 with the following:

SEQ ID NO: 25 is the nucleotide sequence of the full-length acyltransferase clone TAX2.

Please replace the paragraph at page6, lines 7-8 with the following:

SEQ ID NO: 26 is the deduced amino acid sequence of the full-length acyltransferase clone TAX2.

C6

Please replace the paragraph at page 6, lines 9-10 with the following:

SEQ ID NO: 27 is the nucleotide sequence of the full-length acyltransferase clone TAX1.

C6 Cont

Please replace the paragraph at page 6, lines 11-12 with the following:

SEQ ID NO: 28 is the deduced amino acid sequence of the full-length acyltransferase clone TAX1.

Please replace the paragraph at page 7, lines 7-8 with the following:

SEQ ID NO: 44 is the nucleotide sequence of the full-length acyltransferase clone TAX6.

Please replace the paragraph at page 7, lines 9-10 with the following:

SEQ ID NO: 45 is the deduced amino acid sequence of the full-length acyltransferase clone TAX6.

C7

Please replace the paragraph at page 7, lines 14-15 with the following:

SEQ ID NO: 49 is the nucleotide sequence of the full-length acyltransferase clone TAX5.

Please replace the paragraph at page 7, lines 16-17 with the following:

SEQ ID NO: 50 is the deduced amino acid sequence of the full-length acyltransferase clone TAX5.

Please replace the paragraph at page 7, lines 18-19 with the following:

SEQ ID NO: 51 is the nucleotide sequence of the full-length acyltransferase clone TAX7.

Please replace the paragraph at page 7, lines 20-21 with the following:

SEQ ID NO: 52 is the deduced amino acid sequence of the full-length acyltransferase clone TAX7.

Please replace the paragraph at page 7, lines 22-23 with the following:

SEQ ID NO: 53 is the nucleotide sequence of the full-length acyltransferase clone TAX10.

Please replace the paragraph at page7, lines 24-25 with the following:

SEQ ID NO: 54 is the deduced amino acid sequence of the full-length acyltransferase clone TAX10.

Please replace the paragraph at page 7, lines 26-27 with the following:

SEQ ID NO: 55 is the nucleotide sequence of the full-length acyltransferase clone TAX12.

C7

Please replace the paragraph at page 7, lines 28-29 with the following:

SEQ ID NO: 56 is the deduced amino acid sequence of the full-length acyltransferase clone TAX12.

Please replace the paragraph at page 7, lines 30-31 with the following:

SEQ ID NO: 57 is the nucleotide sequence of the full-length acyltransferase clone TAX13.

Please replace the paragraph at page 8, lines 1-2 with the following:

SEQ ID NO: 58 is the deduced amino acid sequence of the full-length acyltransferase clone TAX13.

Please replace the paragraph at page 8, lines 5-10 with the following:

Figure 1: Enzymatic reactions of the Taxol™ pathway indicating cyclization of

C8 geranylgeranyl diphosphate to taxa-4(5),11(12)-diene (not shown), followed by hydroxylation

and double bond rearrangement to form taxa-4(2), 11(12)-dien-5α-ol, followed by acetylation to taxa-4(20),11(12)-dien-5α-yl acetate. The acetate is further converted to 10-deacetylbaccatin III, baccatin III, and Taxol™. In the figure, "a" denotes the activities of taxadiene synthase and taxadiene-5α-hydroxylase (in that order); "b" denotes taxadien-5α-ol acetyl transacylase; and "c" – "e" denote several subsequent steps.

C8

Please replace the paragraph at page 9, lines 1-18 with the following:

Figure 5 shows data obtained from a coupled gas chromatographic-mass spectrometric (GC-MS) analysis of the biosynthetic taxadien-5α-yl acetate formed during the incubation of taxadien-5α-ol with soluble enzyme extracts from isopropyl β-D-thiogalactoside (IPTG)-induced *E. coli* JM109 cells transformed with full-length acyltransferase clones TAX1 and TAX2. Panels **A** and **B** show the respective GC and MS profiles of authentic taxadien-5α-ol; panels **C** and **D** show the respective GC and MS profiles of authentic taxadien-5α-yl acetate; panel **E** shows the GC profile of taxadien-5α-ol (11.16 minutes), taxadien-5α-yl acetate (11.82 minutes), dehydrated taxadien-5α-ol ("TOH-H_2O" peak), and a contaminant, bis-(2-ethylhexyl)phthlate ("BEHP" peak, a plasticizer, CAS 117-81-7, extracted from buffer) after incubation of taxadien-5α-ol and acetyl coenzyme A with the soluble enzyme fraction derived from *E. coli* JM109 transformed with the full-length clone TAX1. Panel **F** shows the mass spectrum of biosynthetically formed taxadien-5α-yl acetate by the recombinant enzyme (11.82 minute peak in GC profile Panel E); panel **G** shows the GC profile of the products generated from taxadien-5α-ol and acetyl coenzyme A by incubation with the soluble enzyme fraction derived from *E. coli* JM109 cells transformed with the full-length clone TAX2 (note the absence of taxadien-5α-yl acetate indicating that this clone is inactive in the transacylase reaction).

C9

Please replace the paragraph at page 15, lines 9-16 with the following:

The National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST™, Altschul et al.. *J. Mol. Biol.* **215**:403-410, 1990) is available from several sources, including the National Center for Biotechnology Information (NCBI, Bethesda, MD) and on the Internet, for use in connection with the sequence-analysis programs blastp, blastn, blastx, tblastn and tblastx. BLAST™ can be accessed at the NCBI online site under the

C10

C10 "BLAST" heading. A description of how to determine sequence identity using this program is available at the NCBI online site under the "BLAST" heading and "BLAST overview" subheading.

Please replace the paragraph at page 16, lines 26-33 with the following:

C11 Biochemical studies have indicated that the third specific intermediate of the Taxol™ biosynthesis pathway is taxa-4(20),11(12)-dien-5α-yl acetate, because this metabolite serves as a precursor of a series of polyhydroxy taxanes en route to the end-product (Hezari and Croteau, *Planta Medica* **63**:291-295, 1997). The responsible enzyme, taxadienol acetyl transacylase, that converts taxadienol to the C5-acetate ester is, thus, an important candidate for cDNA isolation for the purpose of overexpression in relevant producing organisms to increase Taxol™ yield (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999).

Please replace the paragraph at page 19, line 7 with the following:

Accession No. (Seq. Identifier)	Protein Identification No.	Function
AC000103_AT (SEQ ID NO: 59)	g2213627	unknown; from genomic sequence for *Arabidopsis thaliana* BAC F21J9
AC000103_AT (SEQ ID NO: 62)	g2213628	unknown; from genomic sequence for *A. thaliana* BAC F21J9
AF002109_AT (SEQ ID NO: 63)	g2088651	unknown; hypersensitivity-related gene 201 isolog
AC002560_AT (SEQ ID NO: 64)	g2809263	unknown; from genomic sequence for *A. thaliana* BAC F21B7
AC002986_AT (SEQ ID NO: 65)	g3152598	unknown; similarity to C2-HC type zinc finger protein C.e-MyT1 gb/U67079 from *C. elegans* and to hypersensitivity-related gene 201 isolog T28M21.14 from *A. thaliana* BAC
AC002392_AT (SEQ ID NO: 69)	g3176709	putative anthranilate N-hydroxycinnamoyl/benzoyltransferase
AL031369_AT (SEQ ID NO: 70)	g3482975	unknown; putative protein
Z84383_AT (SEQ ID NO: 73)	g2239083	hydroxycinnamoyl:benzoyl-CoA:anthranilate N-hydroxycinnamoyl:benzoyl transferase
Z97338_AT (SEQ ID NO: 74)	g2244896	unknown; similar to HSR201 protein *N. tabacum*
Z97338_AT (SEQ ID NO: 60)	g2244897	unknown; hypothetical protein
AL049607_AT (SEQ ID NO: 61)	g4584530	unknown; putative protein

C12

AF043464_CB (SEQ ID NO: 66)	g3170250	acetyl CoA:benzylalcohol acetyl transferase
Z70521_CM (SEQ ID NO: 72)	g1843440	unknown; expressed during ripening of melon (*Cucumis melo* L.) fruits
AF053307_CR (SEQ ID NO: 68)	g4091808	deacetylvindoline 4-*O*-acetyl transferase
AC004512_DC (SEQ ID NO: 67)	g3335350	unknown; similar to gb/Z84386 anthranilate N-hydroxycinnamoyl/ benzoyltransferase from *Dianthus caryophyllus*
X95343_NT (SEQ ID NO: 71)	g1171577	unknown; hypersensitive reaction in tobacco

C12

Please replace the paragraph at page 23, line 16 through page 24, line 2 with the following:

To determine the identity of the putative taxadienol acetyl transacylase, TAX1, TAX2, and TAX6 were subcloned in-frame into the expression vector pCWori+ (Barnes, *Methods Enzymol.* **272**:3-14, 1996) and expressed in *E. coli* JM109 cells. The transformed bacteria were cultured and induced with isopropyl β-D-thiogalactoside (IPTG), and cell-free extracts were prepared and evaluated for taxadienol acetyl transacylase activity using the previously developed assay procedures (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999). Clone TAX1 (corresponding directly to Probe 1) expressed high levels of taxadienol acetyl transacylase activity (20% conversion of substrate to product), as determined by radiochemical analysis; the product of this recombinant enzyme was confirmed as taxadienyl-5α-yl acetate by gas chromatography-mass spectrometry (GC-MS) (**Figure 5**). Clone TAX2 did not express taxadienol acetyl transacylase activity and was inactive with the [^{3}H]taxadienol and acetyl CoA co-substrates. However, the clone TAX2 may encode an enzyme for a step later in the Taxol™ biosynthetic pathway (TAX2 has been shown to correspond to Probe 2). Neither of the recombinant proteins expressed from TAX1 or TAX2 was capable of acetylating the advanced Taxol™ precursor 10-deacetyl baccatin III to baccatin III. Thus, based on the demonstration of functionally expressed activity, and the resemblance of the recombinant enzyme in substrate

C13

C13 specificity and other physical and chemical properties to the native form, clone TAX1 was confirmed to encode the *Taxus* taxadienol acetyl transacylase.

Please replace the paragraph at page 25, lines 11-22 with the following:

A newly designed isolation and purification method is described below for the preparation of homogeneous taxadien-5α-ol acetyl transacylase from *Taxus canadensis*. The purified protein was N-terminally blocked, thereby requiring internal amino acid microsequencing of fragments generated by proteolytic digestion. Peptide fragments so generated were purified by HPLC and sequenced, and one suitable sequence was used to design a set of degenerate PCR primers. Several primer combinations were employed to amplify a C14 series of twelve related, gene-specific DNA sequences (Probes 1-12). Nine of these gene-specific sequences were used as hybridization probes to screen an induced *Taxus cuspidata* cell cDNA library. This strategy allowed for the successful isolation of eight full-length transacylase cDNA clones. The identity of one of these clones was confirmed by sequence matching to the peptide fragments described above and by heterologous functional expression of transacylase activity in *Escherichia coli*.

Please replace the paragraph at page 26, lines 12-26 with the following:

Unfortunately, the previously described partial protein purification protocol, including an affinity chromatography step, did not yield pure protein for amino acid microsequencing, nor did the protocol yield protein in useful amounts, or provide a sufficiently simplified SDS-PAGE banding pattern to allow assignment of the transacetylase activity to a specific protein (Walker et al., *Arch. Biochem. Biophys.* **364:**273-279, 1999). Furthermore, numerous variations on the affinity chromatography step, as well as the earlier anion exchange and hydrophobic interaction C15 chromatography steps, failed to improve the specific activity of the preparations due to the instability of the enzyme upon manipulation. Also, a five-fold increase in the scale of the preparation resulted in only marginally improved recovery (generally <5% total yield accompanied by removal of >99% of total starting protein). Furthermore, because the enzyme could not be purified to homogeneity, and attempts to improve stability by the addition of polyols (sucrose, glycerol), reducing agents ($Na_2S_2O_5$, ascorbate, dithiothreitol,

C15 β-mercaptoethanol), and other proteins (albumin, casein) were also not productive (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999), this approach had to be abandoned.

Please replace the paragraph at page 27, lines 13-23 with the following:

The soluble enzyme fraction was subjected to ultrafiltration (DIAFLO™ YM 30 membrane, Millipore, Bedford, Massachusetts) to concentrate the fraction from 200 mL to 40 mL and to selectively remove proteins of molecular weight lower than the taxadien-5α-ol acetyl transacylase (previously established at 50,000 Da in Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999). Using a peristaltic pump, the concentrate (40 mL) was applied (2 mL/minute) to a column of *O*-diethylaminoethylcellulose (2.8 X 10 cm, Whatman DE-52, Fairfield, New Jersey) that had been equilibrated with "equilibration buffer" (30 mM HEPES buffer (pH 7.4) containing 3 mM DTT). After washing with 60 mL of equilibration buffer to remove unbound material, the proteins were eluted with a step gradient of the same buffer containing 50 mM (25 mL), 125 mM (50 mL), and 200 mM (50 mL) NaCl.

C16

Please replace the paragraph at page 27, lines 24-29 with the following:

The fractions were assayed as described previously (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999), and those containing taxadien-5α-ol acetyl transacylase activity (125-mM and 200-mM fractions) were combined (100 mL, ~160 mM) and diluted to 5 mM NaCl (160 mL) by ultrafiltration (DIAFLO™ YM 30 membrane, Millipore, Bedford, Massachusetts) and repeated dilution with 30 mM HEPES buffer (pH 7.4) containing 3 mM DTT.

C17

Please replace the paragraph at page 29, lines 4-14 with the following:

The purified protein from multiple preparations as described above (>95% pure, ~100 pmol, 50 μg) was subjected to preparative SDS-PAGE (Laemmli, *Nature* **227**:680-685, 1970). The protein band at 50 kDa, corresponding to the taxadienol acetyl transacylase, was excised. Whereas treatment with V8 protease or treatment with cyanogen bromide (CNBr) failed to yield peptides suitable for sequencing, *in situ* proteolysis with endolysC (Caltech Sequence/Structure Analysis Facility, Pasadena, CA) and trypsin (Fernandez et al., *Anal. Biochem.* **218**:112-118, 1994) yielded a number of peptides, as determined by HPLC, and several of these were

C18

C18 separated, verified by mass spectrometry (Fernandez et al., *Electrophoresis* **19**:1036-1045, 1998), and subjected to Edman degradative sequencing, from which five distinct and unique amino acid sequences (designated SEQ ID NOs: 29-33) were obtained (**Figure 2**).

Please replace the paragraph at page 29, line 18 through page 30, line 8 with the following:

A cDNA library was constructed from mRNA isolated from *T. cuspidata* suspension culture cells that had been induced to maximal Taxol™ production with methyl jasmonate for 16 hours. An optimized protocol for the isolation of total RNA from *T. cuspidata* cells was developed empirically using a buffer containing 100 mM Tri-HCl (pH 7.5), 4 M guanidine thiocyanate, 25 mM EDTA and 14 mM β-mercaptoethanol. Cells (1.5 g) were disrupted at 0-4°C using a Polytron™ ultrasonicator (Kinematica AG, Switzerland; 4 X 15 second bursts at power setting 7), the resulting homogenate was adjusted to 2% (v/v) Triton X-100 and allowed to stand 15 minutes on ice. An equal volume of 3 M sodium acetate (pH 6.0) was then added, and the mixed solution was incubated on ice for an additional 15 minutes, followed by centrifugation at 15,000 *g* for 30 minutes at 4°C. The resulting supernatant was mixed with 0.8 volume of isopropanol and allowed to stand on ice for 5 minutes, followed by centrifugation at 15,000 *g* for 30 minutes at 4°C. The resulting pellet was dissolved in 8 mL of 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA, adjusted to pH 7.0 by addition of 2 mL of 2 M NaCl in 250 mM MOPS buffer (pH 7.0), and total RNA was recovered by passing this solution over a nucleic acid isolation column (Qiagen, Valencia, California) following the manufacturer's instructions. Poly(A)+ mRNA was then purified from total RNA by chromatography on oligo(dT) beads (Oligotex™ mRNA Kit, Qiagen), and this material was used to construct a library using the λZAPII™ cDNA synthesis kit and Gigapack™ III gold packaging kit from Stratagene, La Jolla, California, by following the manufacturer's instructions.

C19

Please replace the paragraph at page 32, lines 13-29 with the following:

C20 The identification of TAX1 (SEQ ID NO: 27) and TAX2 (SEQ ID NO: 25) was accomplished using 1 μg of Probe 1 (SEQ ID NO: 1) that had been amplified by PCR, the resulting amplicon was gel-purified, randomly labeled with [α-^{32}P]CTP (Feinberg and Vogelstein, *Anal. Biochem.* **137**:216-217, 1984), and used as a hybridization probe to screen membrane lifts of 5 X 10^5 plaques grown in *E. coli* XL1-Blue MRF'. Phage DNA was cross-

linked to the nylon membranes by autoclaving on fast cycle 3-4 minutes at 120°C. After cooling, the membranes were washed 5 minutes in 2 X SSC, then 5 minutes in 6 X SSC (containing 0.5% SDS, 5 X Denhardt's reagent, 0.5 g Ficoll (Type 400, Pharmacia, Piscataway, New Jersey), 0.5 g polyvinylpyrrolidone (PVP-10), and 0.5 g bovine serum albumin (Fraction V, Sigma, Saint Louis, Missouri) in 100 mL total volume). Hybridization was then performed for 20 hours at 68°C in 6 X SSC, 0.5% SDS and 5 X Denhardt's reagent. The nylon membranes were then washed two times for 5 minutes in 2 X SSC with 0.1% SDS at 25°C, and then washed 2 X 30 minutes with 1 X SSC and 0.1% SDS at 68°C. After washing, the membranes were exposed for 17 hours to Kodak (Rochester, New York) XAR film at -70°C (Sambrook et al. (ed.), *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, cold Spring Harbor Laboratory Press, cold Spring Harbor, NY, 1989).

C20

Please replace the paragraph at page 34, lines 11-20 with the following:

Isolated transformants for each full-length insert are grown to A_{600} = 0.5 at 37°C in 50 mL Luria-Bertani medium supplemented with 50 μg ampicillin/mL, and a 1-mL inoculum added to a large scale (100 mL) culture of Terrific Broth (6 g bacto-tryptone, DIFCO Laboratories, Spark, Maryland, 12 g yeast extract, EM Science, Cherryhill, New Jersey, and 2 mL glycerol in 500 mL water) containing 50 μg ampicillin/mL and thiamine HCl (320 mM) and grown at 28°C for 24 hours. Approximately 24 hours after induction with 1 mM isopropyl β-D-thiogalactoside (IPTG), the bacterial cells are harvested by centrifugation, disrupted by sonication in assay buffer consisting of 30 mM potassium phosphate (pH 7.4), or 25 mM MOPSO (pH 7.4), followed by centrifugation to yield a soluble enzyme preparation that can be assayed for transacylase activity.

C21

Please replace the paragraph at page 34, lines 24-31 with the following:

A specific assay for acetyl CoA:taxa-4(20),11(12)-dien-5α-ol *O*-acetyl transacylase has been described previously (Walker et al., *Arch. Biochem. Biophys.* **364**:273-279, 1999, herein incorporated by reference). Generally the assay for taxoid acyltransferases involves the CoA-dependent acyl transfer from acetyl CoA (or other acyl or aroyl CoA ester) to a taxane alcohol, and the isolation and chromatographic separation of the product ester for confirmation of

C22